# Identification and Biochemical Characterization of Small-Molecule Inhibitors of *Clostridium botulinum* Neurotoxin Serotype A[∇][§]

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An integrated strategy that combined in silico screening and tiered biochemical assays (enzymatic, in vitro, and ex vivo) was used to identify and characterize effective small-molecule inhibitors of *Clostridium botulinum* neurotoxin serotype A (BoNT/A). Virtual screening was initially performed by computationally docking compounds of the National Cancer Institute (NCI) database into the active site of BoNT/A light chain (LC). A total of 100 high-scoring compounds were evaluated in a high-performance liquid chromatography (HPLC)-based protease assay using recombinant full-length BoNT/A LC. Seven compounds that significantly inhibited the BoNT/A protease activity were selected. Database search queries of the best candidate hit [7-((4-nitro-anilino)(phenyl)methyl)-8-quinolinol (NSC 1010)] were performed to mine its nontoxic analogs. Fifty-five analogs of NSC 1010 were synthesized and examined by the HPLC-based assay. Of these, five quinolinol derivatives that potently inhibited both full-length BoNT/A LC and truncated BoNT/A LC (residues 1 to 425) were selected for further inhibition studies in neuroblastoma (N2a) cell-based and tissue-based mouse phrenic nerve hemidiaphragm assays. Consistent with enzymatic assays, in vitro and ex vivo studies revealed that these five quinolinol-based analogs effectively neutralized BoNT/A toxicity, with CB 7969312 exhibiting ex vivo protection at 0.5 μM. To date, this is the most potent BoNT/A small-molecule inhibitor that showed activity in an ex vivo assay. The reduced toxicity and high potency demonstrated by these five compounds at the biochemical, cellular, and tissue levels are distinctive among the BoNT/A small-molecule inhibitors reported thus far. This study demonstrates the utility of a multidisciplinary approach (in silico screening coupled with biochemical testing) for identifying promising small-molecule BoNT/A inhibitors.

Botulinum neurotoxins (BoNTs), produced by the anaerobic, gram-positive bacterial species *Clostridium botulinum*, *C. baratii*, and *C. butyricum*, consist of seven immunologically distinct serotypes (A to G). BoNTs are synthesized as ~150-kDa single-chain protoxins that are posttranslationally processed by proteolytic cleavage to form a disulfide-linked dimer composed of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) (27, 31, 35, 36). The HC comprises a 50-kDa C-terminal domain (Hc) that participates in the binding of toxin to productive ectoacceptors on the cell surfaces of peripheral cholinergic nerve cells (3). Toxin is taken up into the cell by receptor-mediated endocytosis (4), and the 50-kDa N-terminal domain (Hn) of the HC facilitates the translocation of the LC across an endosomal membrane into the cytosol of the nerve cell (30). The LC is a zinc-dependent endopeptidase that cleaves and inactivates SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins SNAP-25, VAMP/synaptobrevin, and syntaxin (31, 36). SNARE proteins are essential for exocytosis of neurotransmitter, and cleavage of a SNARE protein(s) by BoNT inhibits the release of acetylcholine from synaptic terminals, leading to neuromuscular paralysis or botulism (31, 35).

Worldwide, ~1,000 cases of human BoNT poisoning, predominantly caused by serotypes A and B, are reported yearly (17). In spite of advances in food production and storage/handling processes, cases of food-borne botulism persist, including a massive outbreak in Thailand (26) and the recent U.S. botulism scare associated with canned chili and other products (2, 28).

Therapy for botulism consists of immunological intervention for neutralization and clearance of toxin from the circulation and supportive care, which may include intubation and ventilatory assistance. However, while antibody therapy can be very effective, it has several limitations, including limited availability, lot-to-lot potency variability, and a short window of application. Since small molecules can be potentially used to treat pre- and postexposure BoNT intoxication, research efforts to identify these antagonists have dramatically increased in recent years. However, the discovery and development of BoNT/A small-molecule inhibitors have long challenged researchers. Part of the difficulty in this endeavor can be attributed to the unusually large peptide substrate-enzyme interface (8) that requires a small molecule with high affinity to effectively block

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**14. ABSTRACT**

**Botulinum neurotoxins (BoNTs) comprise a family of seven immunologically distinct proteins synthesized primarily by strains of Clostridium botulinum. BoNTs are the most lethal biological substances known, and are listed as category A agents by the Centers for Disease Control and Prevention. BoNTs are the etiological agents of botulism, an intoxication characterized by peripheral neuromascular blockade and flaccid paralysis. Currently, no effective therapeutics exists to counter botulism. Small-molecule inhibitors of BoNTs can serve as both prophylactics and post-exposure therapeutics. Methodology/Principal Findings: In this study, we report on five novel small-molecule BoNT/A inhibitors that have been discovered by using structure-based virtual screening (VS) in conjunction with tiered screening assays (protease activity, cell culture, and ex vivo mouse phrenic nerve hemidiaphragm assays). The National Cancer Institute (NCI) database that contains >250,000 small molecules was searched by VS against the crystal structure of BoNT/A light chain (LC; PDB code: 1E1H). Compounds identified as hits and their analogs were subjected to HPLC-based assays against LC and BoNT/A (Hall). The top five candidates that effectively inhibited both the LC and the toxin were selected, and further advanced to cellular and tissue-based assays intended to mimic BoNT exposure. These five compounds (at 15 mM) protected N2a cells from BoNT/A-mediated cleavage of SNAP-25, and at low micromolar concentrations, delayed the toxin-induced paralytic time in mouse phrenic nerve hemidiaphragms by at least threefold. Conclusions/Significance: The inhibition of the protease activity of both LC and holotoxin, as well as the low micromolar protection of N2a cells and hemidiaphragms by these five leads are unique among the many recently reported small-molecule inhibitors of BoNT/A. These findings suggest that these lead compounds have high potential for use in the development of therapeutics for botulism, and demonstrate the utility of a systematic, hierarchical screening strategy to identify effective candidate BoNT inhibitors**

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**Clostridium botulinum, neurotoxin, serotype A, small-molecule inhibitors**

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substrate binding (47). Moreover, the BoNT toxin and its domains show considerable conformational flexibility, making design of effective inhibitors complicated. Despite these challenges, a number of papers have been published on the initial steps for discovery and development of inhibitors of BoNT/A protease activity by use of different approaches. Using high-throughput screening of the National Cancer Institute (NCI) Diversity Set, as well as a series of 4-aminoquinolines, Burnett et al. (11) identified several small-molecule inhibitors of BoNT/A, from which a common pharmacophore was predicted using molecular modeling (9). Similarly, a high-throughput screen of a library of hydroxamates (6) resulted in the selection of 4-dichlorocinnamic hydroxamate as a lead structure for further development (5). Capková et al. (12) structurally modified 2,4-dichlorocinnamic acid hydroxamate to improve its potency. On the other hand, a computational screen of 2.5 million compounds resulted in the identification of an inhibitor with a $K_i$ of 12 μM (32), but this value was later invalidated (47). Computer-aided optimization of this inhibitor resulted in an analog that showed a twofold improvement in inhibitory potency and displayed competitive kinetics by chelating the active-site zinc atom (47). Though the above-mentioned approaches have resulted in the identification of a number of small-molecule BoNT/A inhibitors, no compound has yet advanced to preclinical development. The majority of these leads have been demonstrated to be effective only in enzymatic assays (11, 12, 29, 32, 47). Only a few small molecules have been tested in cell-based assays (5, 9, 15) that involved mixing the compound with the toxin, and not by preloading the inhibitor. To date, none of the recently identified BoNT/A inhibitors has been tested in a tissue-based system, yet two compounds were reported to have minimal in vivo activity (15). In this study, we report the identification of potent quinolinol-based BoNT/A small-molecule inhibitors by using an integrated strategy that combined in silico screening and successive biochemical tests, including enzymatic (high-performance liquid chromatography [HPLC]-based), cell-based, and tissue-based assays.

## MATERIALS AND METHODS

**Materials.** Initial test compounds were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD); Sigma-Aldrich (St. Louis, MO); and Chembridge (CB) Corporation (San Diego, CA). Compounds that passed the preliminary HPLC screening were synthesized and purified by GLSynthesis, Inc. (Worcester, MA). The chemical structure and purity (>98%) of these analogs were verified and confirmed by liquid chromatography-mass spectrometry and nuclear magnetic resonance prior to use in subsequent assays. The molecular weights of the compounds were confirmed by mass spectrometry. All compounds tested were racemic mixtures.

BoNT/A peptide inhibitor (Ac-CRATKML-NH₂) was purchased from EMD Chemicals, Inc. (La Jolla, CA). Recombinant full-length BoNT/A and BoNT/B LCs were prepared according to procedures previously described (20, 24) and were >97% pure based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The cloning, expression, and purification of recombinant LC for the type E neurotoxin (rELC; residues 1 to 423) and truncated type A LC (tALC; residues 1 to 425) will be described elsewhere. Briefly, rELC with a C-terminal His₆ tag and tALC were cloned and expressed in *Escherichia coli* (pET24a+/BL21(DE3)). rELC was purified by affinity chromatography, followed by anion-exchange chromatography. Purification of tALC involved a three-step ion-exchange chromatography using Poros HS, Poros HQ, and Source 15S columns. The purity levels of rELC and tALC exceeded 90% and 97%, respectively, as judged by SDS-PAGE. Protein concentration was measured by bicinchoninic acid, using bovine serum albumin as a standard.

BoNT/A (Hall strain) was obtained from Metabiologics (Madison, WI). The specific toxicity of the toxin was $2.4 \times 10^8$ mouse intraperitoneal 50% lethal dose/mg of protein, as determined by a toxin titration procedure described previously (25). Synthetic peptides used as substrates for the HPLC assays were custom synthesized to >98% purity by Quality Controlled Biochemicals (Hopkinton, MA). The Alliance HPLC System (2695 XE separation module and 2996 photodiode array detector) and the Empower/Millenium software program were from Waters (Milford, MA). HPLC columns (Hi-Pore C₁₈; 0.45 by 25 cm) were obtained from Bio-Rad Laboratories (Hercules, CA). Anti-SNAP-25 mouse monoclonal immunoglobulin G₁ (SMI-81) was from CRP, Inc. (Berkeley, CA), and goat anti-mouse horseradish peroxidase-conjugated antibody was from KPL, Inc. (Gaithersburg, MD). Cell culture media and reagents were from Lonza (Walkersville, MD). The enhanced chemiluminescence advance Western blotting detection kit was from GE Healthcare (Piscataway, NJ). Tyrode's buffer was purchased from Sigma (St. Louis, MO).

**Virtual screening of BoNT/A inhibitors.** The three-dimensional structure of BoNT/A LC (Protein Data Bank [PDB] code 1E1H) (39) obtained from the PDB was used for virtual screening since it was the only suitable available crystal structure at the time the in silico screening was performed. One of the protomers was removed to vacate the active site. All water molecules were removed. We added hydrogens and all-atom Kollman charges by using the BIOPOLYMER module from SYBYL. The three-dimensional structures of small molecules for docking were generated using Concord as implemented in SYBYL. DOCK 4.0 (16) was used for docking. Zinc parameters were optimized using the same training set as that described by Hu et al. (23). In all cases, the scoring grids were defined to include the whole active site around the Zn. Ligand fitting with DOCK was employed using anchor-first docking with matching receptor sites and using 25 peripheral seeds, 500 orientations, and uniform sampling. Anchors were first minimized, followed by layer 2 and the whole ligand. Ten thousand minimization steps were done for further refinement. All-atom representation and Gasteiger-Marsili empirical atomic partial charges were used for the ligands.

**HPLC-based BoNT/A and BoNT/B LC protease assays.** Selected compounds from virtual screening were tested in HPLC-based BoNT/A LC and BoNT/B LC enzymatic assays as described previously (21, 37). The BoNT/A LC assay mixture contained 50 mM HEPES (pH 7.3), 0.8 mM substrate corresponding to residues 187 to 203 of SNAP-25 (Ac-SNKTRIDEANQRATKML-NH₂) (37), test compound dissolved in dimethyl sulfoxide (DMSO) at 10× the final assay concentration, and 4.5 to 6.0 μg/ml (110 to 140 nM) BoNT/A LC. The BoNT/B LC reaction mixture contained 50 mM HEPES buffer (pH 7.3), 0.4 mM substrate corresponding to residues 60 to 94 of human VAMP-2 (Ac-LSELDDRADALQAGASQF ETSAAKLKRKYWWKNLK-NH₂) (42), 1 mM dithiothreitol, test compound in DMSO, and 1.5 to 2 μg/ml (30 to 40 nM) BoNT/B LC. In control assays, the test compound was replaced by DMSO. The reaction mixture was immediately mixed upon addition of the LC and incubated at 37°C for 5 min. Assays were stopped by acidification with 90 μl of 0.7% trifluoroacetic acid. The amounts of uncleaved substrate and products were then measured after separation by reverse-phase HPLC.

**Determination of the IC₅₀.** Fifty-percent inhibitory concentration (IC₅₀) values for BoNT/A LC were calculated from nine concentrations of compound by a log-probit analysis program, using the statistical software program GraphPad Prism 4 (GraphPad Software, La Jolla, CA).

**SNAP-25 gel cleavage assay by rELC.** Recombinant SNAP-25 (13 μM; List Biological Laboratories, Inc., Campbell, CA) was incubated with 6 μM rELC and 200 μM compound (dissolved in DMSO at 10× the final assay concentration) and incubated at 37°C for 30 min. The positive control had rELC and DMSO. Reactions were stopped by adding SDS-PAGE buffer and heating for 5 min at 70°C. Samples were run on 12% Nu-PAGE Bis Tris gels (Invitrogen, Carlsbad, CA) and stained by Simply Blue (Invitrogen, Carlsbad, CA).

**CPA assay.** The carboxypeptidase A (CPA) plate assay was performed according to manufacturer's (Sigma, St. Louis, MO) recommendation. Briefly, the mixture contained sample reaction buffer, ultrapure water, various concentrations of compounds dissolved in DMSO, and CPA. The positive control had CPA and DMSO, and the negative control contained CPA and CPA inhibitor from a potato tuber. The plate was incubated for 5 min at 25°C, and the reaction was stopped by the addition of stop solution. The absorption at 350 nm was read, and CPA activity was calculated. Percent inhibition was determined by comparing the CPA activity produced in the reaction mixtures with CPA alone and in the reaction mixtures containing CPA and the test compound. Values shown are averages of results from two independent determinations, each done in triplicate.

**Cell culture assay.** The cell culture assay was based on the procedures of Yowler et al. (49) and Boldt et al. (7). Briefly, cells of the murine cholinergic neuroblastoma cell line Neuro-2a (ATCC CCL-131) were incubated in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 10 mM HEPES, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml of

streptomycin in a 75-cm$^2$ cell culture flask at 37°C in an atmosphere of 5% $CO_2$ and 95% air. Upon reaching 70 to 80% confluence, the medium was removed and the cells were washed with Dulbecco's phosphate-buffered saline without $Ca^{2+}$ or $Mg^{2+}$. Cells were pelleted, diluted to $1.5 \times 10^5$ cells/ml, and plated in six-well cell culture plates (2 ml/well). After incubation for 48 h, the medium was removed and replaced with serum-free medium, and the cells were grown for an additional 24 h.

The inhibitor (0.6 to 0.9 μl of the initial stock of 16.7 mM in 100% DMSO) was mixed with 1.5 μl BoNT/A (1 mg/ml) in a total volume of 2.4 μl and incubated for 30 min at 37°C. The toxin-DMSO (control) or toxin inhibitor mixture was added to 1 ml of EMEM without fetal bovine serum to bring the final concentration of BoNT/A toxin to 10 nM. The concentration of BoNT/A was calibrated to produce ≥50% cleavage of the substrate SNAP-25 in a 24-h incubation at 37°C. In all samples, including controls, the final concentration of DMSO was ≤0.09%.

At the end of the incubation, the medium was removed, and the cells were lysed with CelLytic M (Sigma, St. Louis, MO). Samples were run on 12% NuPAGE Bis-Tris gels (Invitrogen) and subjected to Western blot analysis using anti SNAP-25 monoclonal antibody (CRP, Inc.), followed by goat anti-mouse horseradish peroxidase-conjugated secondary antibody (KPL, Inc.). Samples were visualized using an enhanced chemiluminescence advance Western blotting detection kit (GE Healthcare). Signals were quantitated using the UN-SCAN-IT *gel* software program (Silk Scientific, Orem, UT). Data presented are representative of results from three independent assays.

**MPNHDA.** The mouse phrenic nerve hemidiaphragm assay (MPNHDA) was conducted based on the procedures of Sheridan et al. (40). Female CD-1 mice (20 to 25 g) were euthanized with $CO_2$, and their diaphragms with attached phrenic nerves were removed. The diaphragms were then divided into two hemidiaphragms, with each section complete with a phrenic nerve and myoneural junction. Each hemidiaphragm was attached to an isometric force transducer (Fohr Medical Instruments, Seeheim, Germany), and its phrenic nerve was secured to a stimulating electrode. The nerve-muscle preparations were immersed in separate 10-ml tissue baths containing Tyrode's buffer (1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.7 mM KCl, 137 mM NaCl, 0.4 mM $NaH_2PO_4$, 12 mM $NaHCO_3$, and 6 mM glucose), pH 7.2 to 7.4 (Sigma, St. Louis, MO). A mixture of 95% $O_2$-5% $CO_2$ gas was passed through the Tyrode's buffer. The tissue baths were kept at 37°C.

Each phrenic nerve was stimulated with single supramaximal pulses (SD9 Stimulators Grass Instruments, Warwick, RI) through a Powerlab/4sp and Bridge Amp relay (ADInstruments, Inc., Colorado Springs, CO) with a 0.3-ms duration at 0.03 Hz. The twitch tensions were digitally recorded by Chart software (ADInstruments, Inc., Colorado Springs, CO). After acclimation to the tissue baths, the tissue preparations were run for 20 to 30 min for baseline measurements. The inhibitor (dissolved in DMSO at 2× the final assay concentration) was mixed with 60 pM BoNT/A (Metabiologics, Madison, WI) in 5 ml of Tyrode's buffer and incubated for 15 to 20 min at 37°C. After baseline stabilization, the toxin-inhibitor mixture was added to a 10-ml bath with an additional 5 ml of Tyrode's buffer, bringing the final concentration of BoNT/A toxin to 30 pM. The concentration of BoNT/A neurotoxin was previously calibrated to induce a 50% loss of twitch tension in approximately 60 min. In all samples, including the controls, the final concentration of DMSO was 0.3%.

For each experiment, four tissue baths were used. One bath was the BoNT/A toxin-only control. A second bath was an assay control without toxin or inhibitor. The third and fourth baths contained toxin plus two different concentrations of inhibitor. Adding the toxin or the toxin/inhibitor mixture to the bath initiated the beginning of data collection, which continued for 5 h or until muscle twitch tension ceased.

For all preparations, neurotoxin-induced paralysis was measured as a 50% loss of twitch tension evoked by nerve stimulation. Estimates of statistical significance were based on unpaired two-tailed $t$ test, with a $P$ value of <0.05 considered significant, as previously reported (13, 40, 41). Statistical analysis was performed using SigmaPlot 10 (Systat Software, San Jose, CA).

Procedures used to obtain mouse tissues were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

## RESULTS

**Strategy.** The flow chart shown in Fig. 1 outlines the overall strategy for the identification of small-molecule inhibitors of
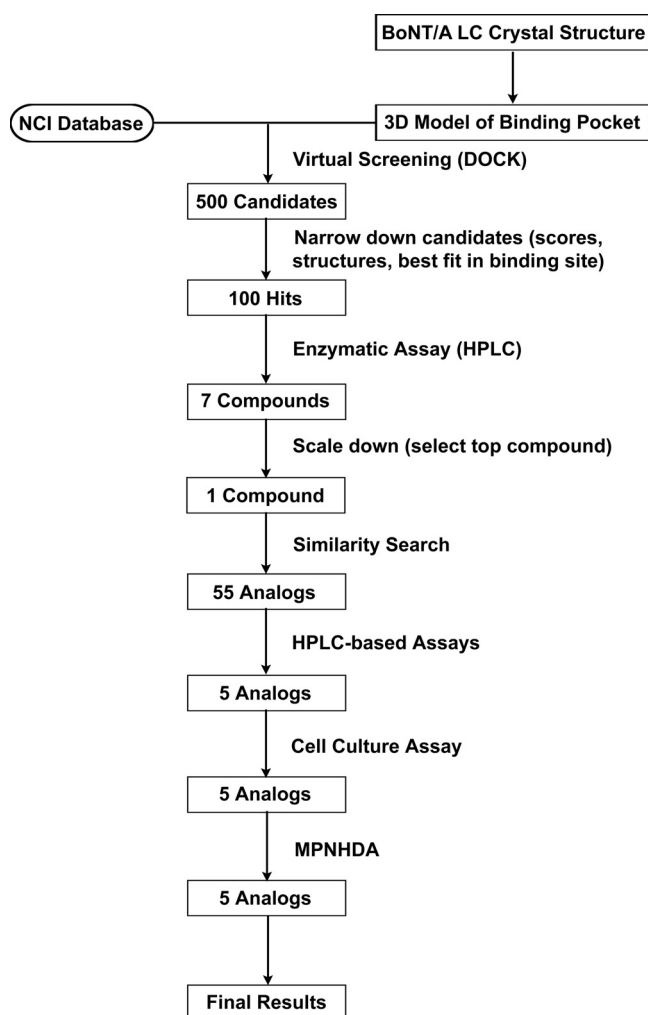


FIG. 1. Integrated experimental flow chart for identification of BoNT/A small-molecule inhibitors.

BoNT/A. We focused on serotype A since it is the most prevalent and well studied among the various serotypes in human intoxication. We used in silico screening to identify BoNT/A inhibitors. Selected compounds were tested for inhibition of the protease activity of BoNT/A LC. Compounds that passed the HPLC screens were advanced to in vitro and ex vivo assays.

**Virtual screening of the NCI database.** The NCI database was chosen for virtual screening on the basis of the following considerations. First, it constitutes the largest freely available public domain chemical structure database, with >250,000 compounds (48). Second, it contains a high number of unique and structurally diverse compounds. Thus, the NCI database provides an opportunity to discover lead compounds (48).

The compounds from the NCI database were docked into the active site in one of the protomers of BoNT/A LC (PDB code 1E1H) (39) after removal of the peptide occupying the active site in the protomer. The top-scoring 500 compounds were evaluated in more detail; the list was narrowed to 100 structurally diverse compounds that interacted well with the active-site Zn and demonstrated a good fit in the BoNT/A LC binding site.
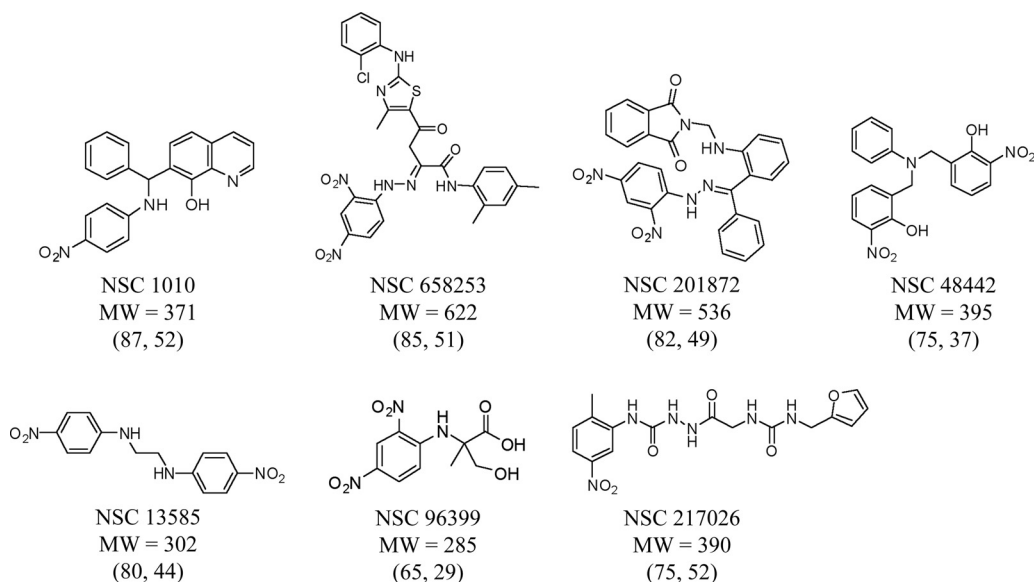
FIG. 2. Structures of selected hits and percent inhibition against recombinant full-length BoNT/A LC (rALC). Structures were obtained from the NCI Developmental Therapeutics Program website (http://dtp.nci.nih.gov/index.html/). Compounds were tested in an HPLC-based assay using recombinant full-length BoNT/A LC (140 nM) in the presence of 0.8 mM 17-mer SNAP-25 peptide substrate (see Materials and Methods for details). Percentages of inhibition at 200 and 20 μM are indicated in parenthesis and were determined by the amounts of peptide substrate cleaved in the presence or absence of inhibitors under the same conditions.

**HPLC-BoNT/A LC protease assay.** To determine the actual effectiveness of the compounds predicted by the virtual screening, an HPLC-based enzymatic assay was performed in the presence and absence of inhibitor at 20 μM and 200 μM using full-length recombinant BoNT/A LC (rALC). The substrate peptide for BoNT/A LC protease activity consisted of residues 187 to 203 of SNAP-25, and BoNT/A LC catalyzes the hydrolysis between residues Q197 and R198 (37). Out of 100 tested, 7 compounds that inhibited rALC by more than 60% and 30% at 200 μM and 20 μM, respectively, were selected (Fig. 2). These compounds had diverse chemical structures, with molecular weights ranging from 285 to 622. Of these seven "hits," the two most potent compounds were NSC 1010 and NSC 658253. In this study, we focused on the quinolinol lead NSC 1010. Selection of this compound was based on the following observations: (i) NSC 1010 was very potent against rALC; (ii) NSC 1010 failed to inhibit BoNT serotype B LC (not shown), suggesting its selectivity for serotype A; (iii) there are quinolinols in clinical trials for Alzheimer's disease and cancer (22, 33, 34); and (iv) quinolinol-based drugs, such as linolasept and vioform (generic name, clioquinol), are available in the market. Thus, this set of information implies that NSC 1010 is a good starting point for BoNT antitoxin development. Work on the remaining six compounds is under way and will be reported separately.

**Screening and testing of analog compounds.** To verify chemical identity, we synthesized NSC 1010 and confirmed its structure and purity. Further evaluation of this compound revealed that it was toxic to neuroblastoma N2a cells in cellular assays at ≥10 μM (data not shown). Thus, we performed similarity searches of the Sigma, Chembridge, and NCI databases to look for nontoxic analogs. Fifty-five analogs were identified and synthesized. Testing of these analogs in HPLC-based assays

demonstrated five analogs (NSC 84094, NSC 84096, CB 7967495, CB 7969312, and CB 7968218) to be more potent than the original hit NSC 1010 and, equally importantly, nontoxic to cells (see below). These five analogs constitute the final compounds that were subsequently characterized in cell- and tissue-based assays. Since these compounds are racemates, efforts are currently under way to separate and evaluate the individual enantiomers. Whether one or both of the enantiomers contributed to the inhibition is not entirely clear, since the docking poses for the two enantiomers of NSC 84096 in the active site of BoNT/A LC were different but gave comparable scores (not shown).

To compare the relative inhibitory potencies of the five analogs, HPLC-based assays were performed using two recombinant forms of BoNT/A LC: full-length BoNT/A LC (rALC) and truncated BoNT/A LC (tALC; residues 1 to 425). Under our assay conditions, rALC was at least four times more active than tALC. The five analogs were very effective against both forms of BoNT/A LC, demonstrating $IC_{50}$ values ranging from 1.6 to 4.7 μM and 1.5 to 5.0 μM for rALC and tALC, respectively (Fig. 3).

Having established that these five analogs were very potent against BoNT/A LC, we next tested their effects on two related BoNT endopeptidases, recombinant full-length BoNT/B LC (rBLC) and BoNT/E LC (rELC). The five compounds showed cross-reactivity against rBLC, with both NSC 84096 and NSC 84094 exhibiting the least inhibition (at 6.9% and 8.8%, respectively) (Table 1). However, none of the compounds (at 240 μM) inhibited rELC in gel cleavage assays (Fig. 4A). Although additional BoNT LC serotypes would need to be tested before these five compounds are concluded to be more selective for BoNT/A, this possibility deserves consideration.

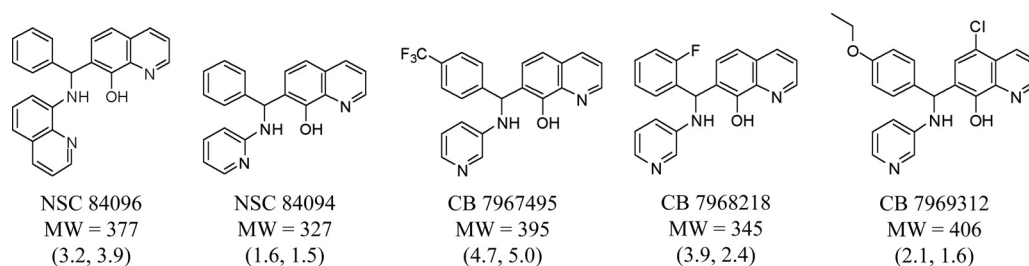Further experimental studies revealed that all five com-

FIG. 3. Structural formula and IC$_{50}$ values of selected analogs against recombinant full-length BoNT/A LC (rALC) and truncated BoNT/A LC (tALC; residues 1 to 425). Structures of NSC compounds were obtained from the NCI Developmental Therapeutics Program (http://dtp.nci.nih .gov/index.html/), and those of CB compounds were from Chembridge screening compounds and building blocks (http://www.hit2lead.com/). Each compound structure was redrawn by using ChemDraw Ultra 11 (http://www.cambridgesoft.com/). All compounds tested were racemates. The structure and purity of these analogs were confirmed by liquid chromatography-mass spectrometry and nuclear magnetic resonance. IC$_{50}$ values for rALC and tALC are indicated in parentheses and were determined from nine concentrations of each inhibitor by using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). rALC (140 nM final concentration) or tALC (620 nM) was incubated with 0.8 mM 17-mer SNAP-25 peptide substrate and various concentrations of inhibitor (dissolved in DMSO at 10× the final concentration) at 37°C for 5 min in 50 mM HEPES, pH 7.3. Reactions were stopped by adding 0.7% trifluoroacetic acid and analyzed by reverse-phase HPLC (see Materials and Methods for details).

pounds (at concentrations up to 20 μM) showed no inhibitory effect on the zinc protease carboxypeptidase (CPA), while the control inhibitor from potato tuber exhibited 100% inhibition at concentrations equal to or greater than 0.187 μM (not shown). This finding precludes the possibility that the inhibition of BoNT/A endopeptidase by these analogs was due to nonspecific chelation.

**Activity in biological systems. (i) Inhibition in a cell-based assay.** To this point, we have shown that five compounds inhibit the enzymatic activity of BoNT/A in vitro. To examine the likelihood that these compounds might be useful antibotulinum therapeutics, we performed studies with other systems. One of these used murine neuroblastoma N2a cells to evaluate the ability of these compounds to protect BoNT/A-mediated cleavage of intracellular SNAP-25. BoNT/A binds to N2a cells, and the translocated LC cleaves SNAP-25 in these cells. The extent of cleavage of SNAP-25 in N2a cells by BoNT/A was determined by Western blot analysis using monoclonal antibodies against SNAP-25. As shown in Fig. 4B, all five lead analogs at 15 μM exhibited complete protection of BoNT/A-mediated SNAP-25. At 10 μM, three of these compounds (CB

7967495, NSC 84094, and CB 7969312) showed near-complete-to-complete inhibition of SNAP-25 cleavage while the other two (NSC 84096 and CB 7968218) afforded 66% and 68% protection, respectively (Fig. 4C).

**(ii) MPNHDA.** Encouraged by these findings, we then examined the efficacy of these analogs at the tissue level by using mouse phrenic nerve hemidiaphragm preparations whose intact neuromuscular junction permits the monitoring of the
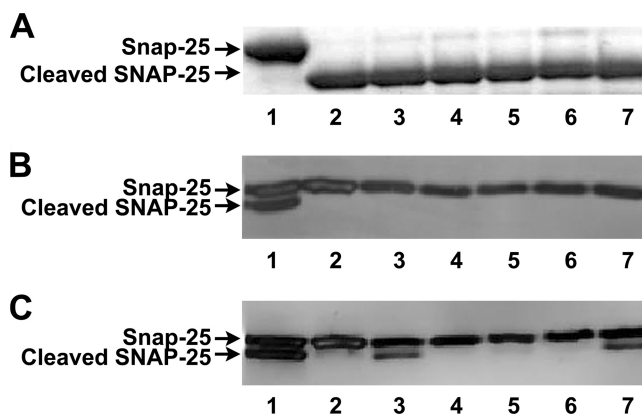
TABLE 1. Percents inhibition of selected small molecules against recombinant BoNT/A light chain (rALC) and BoNT/B light chain (rBLC)[a]

| Compound | % Inhibition against | |
|---|---|---|
| | rALC | rBLC |
| NSC 84096 | 91.9 ± 1.7 | 6.9 ± 2.0 |
| NSC 84094 | 97.4 ± 2.2 | 8.8 ± 0.8 |
| CB 7967495 | 92.7 ± 1.5 | 30.2 ± 2.4 |
| CB 7968218 | 92.8 ± 0.8 | 16.8 ± 2.2 |
| CB 7969312 | 96.2 ± 1.2 | 31.2 ± 3.0 |

[a] HPLC-based protease assays were conducted at 37°C using various inhibitors at a 20 μM final concentration. rALC assay mixtures contained 50 mM HEPES (pH 7.3), 0.8 mM SNAP-25 peptide substrate, test compound in DMSO, and rALC (110 to 140 nM). The assay mixture for rBLC contained 50 mM HEPES (pH 7.3), 1 mM dithiothreitol, 0.4 mM 35-mer VAMP peptide substrate, test compound in DMSO, and rBLC (30 to 40 nM). Inhibitors were diluted into the reaction mixture containing the substrate, followed by the addition of LC (i.e., the inhibitor and LC were not preincubated). Reactions were stopped by acidification by TFA and analyzed by reverse-phase HPLC as indicated in Materials and Methods. Data represent means ± SD of results from two independent assays.



FIG. 4. Efficacy of small-molecules in inhibiting SNAP-25 cleavage. (A) Gel cleavage assay using recombinant BoNT/E LC (rELC). Inhibitors (240 μM) and rELC (6.0 μM) were added to recombinant SNAP-25 (S25; 12.9 μM) and incubated at 37°C for 30 min. The reaction products were analyzed on SDS-PAGE gels. Lane 1, S25 alone; lanes 2 to 7, S25 with rELC (lane 2), NSC 84096 (lane 3), CB 7967495 (lane 4), CB 7969312 (lane 5), NSC 84094 (lane 6), and CB 79698218 (lane 7). Panels B and C. Inhibition of BoNT/A-mediated SNAP-25 cleavage by small-molecules in cell-based assay. N2a cells were grown in EMEM with supplements at 37°C in an atmosphere of 5% CO$_2$ and 95% O$_2$. Upon 70 to 80% confluence, the cells were used to seed a six-well plate and incubated for 48 h. The medium was removed and replaced with serum-free medium. Cells were grown for an additional 24 h with either 10 nM BoNT/A-DMSO or inhibitor with 10 nM BoNT/A. The cells were harvested and the samples were analyzed by Western blotting (see Materials and Methods for details). Efficacy of five small-molecule inhibitors at 15 μM (B) and at 10 μM (C). Lane 1, with BoNT/A; lane 2, without BoNT/A; lanes 3 to 7, BoNT/A with inhibitor NSC 84096 (lane 3), CB 7967495 (lane 4), CB 7969312 (lane 5), NSC 84094 (lane 6), and CB 7968218 (lane 7). These blots represent three independent experiments.

TABLE 2. Effect of small-molecule inhibitors in protecting BoNT/A-induced neuromuscular block in mouse phrenic nerve hemidiaphragm assays[a]

| Inhibitor | Concn (μM) | Avg time to 50% loss of twitch tension (min) |
|---|---|---|
| Without BoNT/A | | >300.00 |
| BoNT/A with: | | 65.70 ± 7.80 |
| NSC 84096 | 20.0 | 216.3 ± 20.2[b] |
| NSC 84094 | 10.0 | 266.3 ± 19.6[b] |
| CB 7967495 | 5.00 | 216.3 ± 25.2[b] |
| CB 7968218 | 10.0 | 281.0 ± 19.0[b] |
| CB 7969312 | 0.50 | 271.0 ± 29.0[b] |
| CRATKML | 20.0 | 52.70 ± 9.30[c] |

[a] The specified concentrations of small-molecule inhibitors and BoNT/A were added to hemidiaphragm preparations, and isometric contractions of the electrically stimulated muscles were recorded and analyzed (for details, see Materials and Methods). The time required to 50% of loss of twitch tension (paralytic half-time) was determined. Controls (with and without BoNT/A) used the same amount of DMSO (0.3% final concentration) as those with inhibitors. Data are means ± SE ($n = 3$ for small molecules and CRATKML; $n = 18$ for groups with BoNT/A and control groups without BoNT/A).

[b] $P$ values of <0.01 (highly significant).

[c] $P$ values of >0.05 (not significant) for comparison with values recorded for the BoNT/A control. Statistical analysis was performed using SigmaPlot 10 (Systat Software, San Jose, CA).

effectiveness of an inhibitor by recording muscle twitch tension. In the ex vivo MPNHDA, the time to onset of neuromuscular block is a concentration-sensitive event, with blockade occurring earlier with higher toxin concentrations (13, 45, 46). Changes in the onset of muscle paralysis with a fixed concentration of toxin are used to indicate the activity of candidate BoNT therapeutics.

Results from MPNHDA experiments demonstrated that applying effective concentrations of compounds significantly delayed ($P < 0.01$) the onset of toxin-induced paralysis (Table 2). While neuromuscular preparations exposed to BoNT/A alone (control) had an average paralytic time of 65.7 ± 7.80 min, those that were exposed to inhibitors at effective concentrations showed times ranging from 216.3 to 281.0 min. The effective concentrations that caused a delay in time to 50% loss of twitch tension for the five inhibitors ranged from 0.5 μM to 20 μM. Moreover, the muscle twitch-tension time courses of the five inhibitors at their effective concentrations during the entire assay period (300 min) were comparable to those observed for the no-toxin control group (Fig. 5). In comparison, the peptide BoNT/A inhibitor CRATKML ($K_i = 1.9$ μM) (38) did not cause a delay in paralytic time at 20 μM and showed muscle twitch-tension time courses that were similar to that of BoNT/A alone (control) (Fig. 5). To our knowledge, this is the first report of BoNT/A small-molecule inhibitors that showed activity in an ex vivo assay.

## DISCUSSION

In this paper, we report the initial identification of seven small-molecule inhibitors of BoNT serotype A by using in silico screening of the NCI database followed by HPLC protease assays. We used a substructure/similarity search to identify analogs of the lead hit NSC 1010 and focused our study on five of its most potent analogs, i.e., NSC 84094, NSC 84096, CB 7967495, CB 7968218, and CB 7969312. Not only were these

five analogs highly effective against the full-length BoNT/A LC in the HPLC-based assays, but they were also very active against a truncated form of BoNT/A LC (residues 1 to 425). The extent of inhibition and the $IC_{50}$ values of these leads were comparable to or even better than those of previously reported small-molecule inhibitors (5, 6, 9–12, 15, 29, 32). As of this date, no small-molecule has been reported as active against both full-length and truncated forms of BoNT/A LC. Currently, it still remains unclear what form of LC exists in the cytosol during the actual BoNT catalysis of SNARE proteins. At this stage in our understanding of BoNT/A inhibition, information on the activity of candidate small-molecule inhibitors against both forms of LC is useful.

The crystal structures of the endopeptidase of different serotypes of BoNT are very similar. The HEXXH motif that is characteristic of the catalytic site of Zn-endopeptidase is conserved. For instance, the active sites of BoNT/A and BoNT/B endopeptidases differ only in two residues: F162 and F193 in BoNT/A correspond to N169 and S200 in BoNT/B (32). Thus, it is conceivable for some BoNT/A inhibitors to be also active against other serotypes. We found that our five analogs exhibited cross-reactivity, albeit reduced, against BoNT/B LC. Such a finding is not unprecedented. The two inhibitors described by Tang et al. (47) were also reported to inhibit BoNT/B LC at concentrations of >20 μM. Interestingly, all of our five compounds (at 240 μM) failed to inhibit BoNT serotype E LC. Although additional serotypes are needed to be tested, this finding seems to suggest that they are more selective to serotype A.
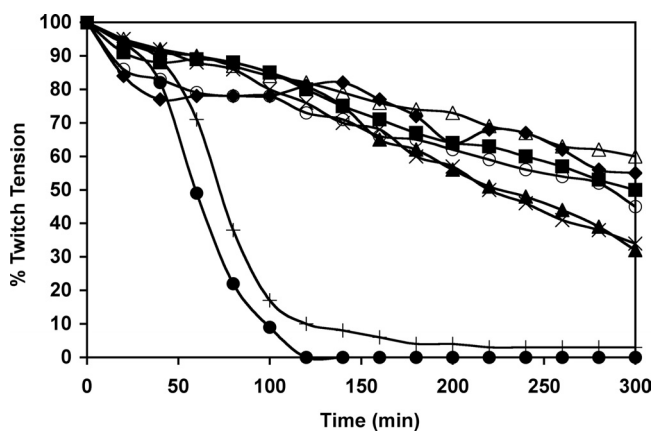


FIG. 5. Effects of BoNT/A small-molecule inhibitors in MPNHDA. Mouse hemidiaphragms were attached to isometric force transducers, and the phrenic nerves were secured to stimulating electrodes. Inhibitors (dissolved in DMSO at 0.3% final assay concentration) were mixed with BoNT/A toxin (30 pM final concentration) and incubated at 37°C for 15 min prior to addition to the bath after baseline stabilization. Twitch tension data collection continued for 5 h or until muscle twitch tension ceased (for details, see Materials and Methods). In all assays, BoNT/A (+) and No Toxin (△) controls were run to demonstrate the difference in twitch tension between BoNT-intoxicated and normal tissues ($n = 18$ for both groups). The twitch tension time courses are the averages of results from triplicate assays. The time courses include only the effective concentration of inhibitor required for maximum protection, except for CRATKML, which was not effective at the tested concentration: CB 7969312 (500 nM) (■), CB 7967495 (5 μM) (▲), NSC 84094 (10 μM) (○), CB 7968218 (10 μM) (◆), NSC 84096 (20 μM) (x), and CRATKML (20 μM) (●).

It is also significant that efficacy of these candidate inhibitors against BoNT/A holotoxin was observed in cellular assays. Cells treated with all five compounds showed protection from the deleterious effects that BoNT/A had on SNAP-25 at concentrations lower than the effective levels of previously reported small-molecule inhibitors (5, 9, 15). Of further importance is the observation that these five compounds exhibited little or no cell toxicity, a characteristic that is highly desirable in the development of a therapeutic drug. Unlike several BoNT/A small-molecule inhibitors that were reported as cytotoxic at >5 μM (15) or >40 μM (9), four of our lead compounds (NSC 84096, CB 7969312, NSC 84094, and CB 7967495) were well tolerated by the cells at concentrations up to 50 μM, and one (CB 7968218) exhibited signs of toxicity (aggregation and detachment of N2a cells from the plate surfaces) only at concentrations above 45 μM. This result adds credence to the potential of these compounds for further development.

Our findings in the tissue-based assay revealed that all five compounds were highly effective in the MPNHDA, significantly delaying the BoNT/A-induced paralytic half-time at least threefold, while the peptide BoNT/A inhibitor CRA TKML was not protective. Among the five lead inhibitors, CB 7969312 was the most effective, at 0.5 μM. This striking observation represents CB 7969312 to be the most potent small-molecule BoNT/A inhibitor reported to date that exhibited activity in a tissue-based assay. While the in vivo mouse bioassay is still considered the gold standard for evaluating the efficacy of candidate BoNT inhibitors, during the early phase of drug discovery and development, the ex vivo MPNHDA has a potential over the in vivo assay in that it requires only two mice per sample tested, and the results can be available within hours.

It should be pointed out that the effectiveness of these inhibitors in the in vitro and ex vivo assays was demonstrated only when the compound was premixed with BoNT/A toxin; thus far, preloading the inhibitor did not protect cells/tissues against BoNT intoxication (data not shown). To our knowledge, no group has been able to provide experimental evidence showing that their inhibitors work in a preloading system. The small-molecule inhibitor that was reported to be active in primary neurons (9) was demonstrated to show a dose-dependent inhibition of SNAP-25 cleavage in a nonpreloading system (cells were pretreated with inhibitor for 45 min followed by incubation with BoNT/A in the continuous presence of inhibitor). Additionally, the inhibitors reported by Eubanks et al. (15) and Boldt et al. (7) were characterized in cell culture assays that involved mixing BoNT/A toxin and various concentrations of inhibitor.

One of the key challenges in the development of small-molecule therapeutics against BoNT is the difficulty involved in delivering the compounds to the neuronal cytosol. BoNTs act intracellularly; their sites of action are the cholinergic nerve cells (31, 35). Hence, inhibitors ought to be specifically and precisely targeted to these neuronal cells to effect internalization. Potential therapeutic applications of small-molecule BoNT/A inhibitors would plausibly necessitate the development of a delivery vehicle system for more-precise localization and appropriate release of compounds into the cytoplasm of the peripheral cholinergic nerve cells.
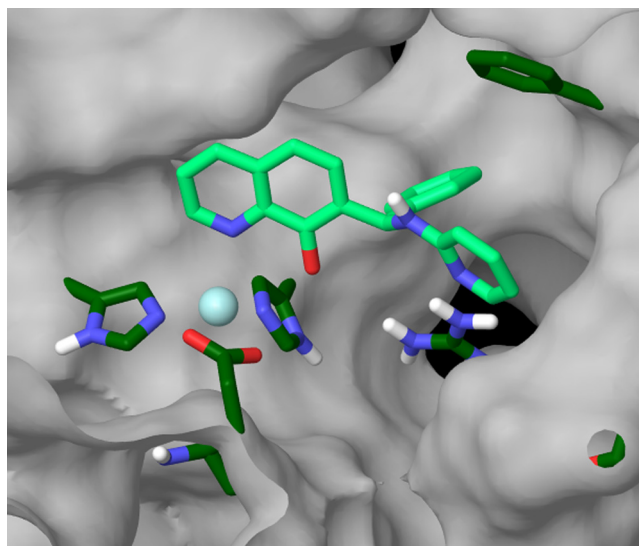


FIG. 6. Binding mode of NSC 84094 into BoNT/ALC substrate binding cleft showing the quinolinol group (light green) interacting with the Zn atom (light blue), while the pyridyl substituent can form hydrogen bond with Arg363. Atom colors: nitrogen (blue), oxygen (red), hydrogen (white), enzyme C atoms (dark green), and inhibitor C atoms (light green).

The small-molecule inhibitors identified in this report have three aromatic groups, one of which is an 8-quinolinol moiety. Quinolinol is known to chelate divalent cations (14) and also has antimicrobial properties (18, 19). Because of potential indiscriminate metal chelation, the 8-hydroxyquinoline motif has been tagged as a "not suitable" functional component of BoNT/A LC inhibitors (11). However, the particular class of quinolinol identified in our study (with the other two ring systems and a secondary amine; see below) displayed specificity for BoNT serotype A and did not inhibit simply by chelating active-site zinc.

The structures of the quinolinol derivatives reported in our paper also contain additional basic moieties including 2-amino or 3-amino pyridine (NSC 84094, CB 7967495, CB 7968218, and CB 7969312). The presence of these structural motifs suggests that these lead inhibitors may interact with the hydrophobic pocket located in the active site of the LC (see below). Adler et al. (1) reported that the quinolinol moiety alone in the presence of zinc did not inhibit the proteolytic activity of BoNT serotypes A and B.

While the mechanism by which these newly identified small-molecules inhibit BoNT/A is still being elucidated, molecular docking provided key insights into the likely binding sites and mode of inhibition. As shown in Fig. 6, NSC 84094 is docked in the large hydrophobic pocket of the BoNT/A LC active site, and its hydroxyquinoline moiety coordinates with zinc. This could explain the importance of this group in inhibiting BoNT/A LC and suggests that the quinolinols inhibit BoNT/A by blocking the active site zinc. Additionally, the pyridyl ring can form a hydrogen bond with Arg363 and may contribute to the specificity and potency of the inhibitor. It should be noted that the crystal structures of the complexes of known small-molecule and peptide inhibitors with BoNT/A LC have shown that chelation to zinc is involved in the binding and inhibition

of the LC in both cases (43, 44). A small-molecule inhibitor reported to have some in vivo efficacy (15) is a zinc chelator (5). Chelation appears to be a necessary but not sole condition of known inhibitors. Further studies are warranted to fully determine the precise mechanism of action of these compounds.

In summary, the five small-molecule compounds reported in this study represent highly potent, nontoxic BoNT/A inhibitors that were identified using an integrated screening strategy. These compounds effectively inhibited the protease activities of both BoNT/A LC (full-length and truncated) and also significantly neutralized BoNT/A holotoxin in N2a cells and hemidiaphragm assays. Such protection at the cellular and tissue levels is particularly important, since previously reported potent BoNT/A LC inhibitors, such as CRATKML, were ineffective under these conditions. Moreover, the effective inhibition by these compounds of BoNT/A LC, but not BoNT/E LC, as well as their reduced efficiency of BoNT/B LC, suggests that these small-molecules preferentially interact with BoNT/A LC. We recognize that these leads are not yet viable drug candidates, and for this type of inhibitor to advance, they need to be optimized, not only for potency but also for other important drug characteristics, including absorption, distribution, metabolism, excretion, and toxicity. Collectively, our results provide a candidate structural class for potential development as well as the blueprint for an effective discovery process that should be applicable to finding inhibitors of other BoNT serotypes.

## ACKNOWLEDGMENTS

## REFERENCES

1. **Adler, M., R. E. Dinterman, and R. W. Wannemacher.** 1997. Protection by the heavy metal chelator N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) against the lethal action of botulinum neurotoxin A and B. Toxicon **35**:1089–1100.
2. **Associated Press.** 24 July 2007, posting date. Castleberry expands food recall over link to botulism outbreak. Fox Entertainment Group, New York, NY. http://www.foxnews.com/story/0,2933,290507,00.html.
3. **Black, J. D., and J. O. Dolly.** 1986. Interaction of $^{125}$I-labeled botulinum neurotoxins with nerve terminals. I. Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. J. Cell Biol. **103**:521–534.
4. **Black, J. D., and J. O. Dolly.** 1986. Interaction of $^{125}$I-labeled botulinum neurotoxins with nerve terminals. II. Autoradiographic evidence for its uptake into motor nerves by acceptor-mediated endocytosis. J. Cell Biol. **103**:535–544.
5. **Boldt, G. E., J. P. Kennedy, and K. D. Janda.** 2006. Identification of a potent botulinum neurotoxin A protease inhibitor using in situ lead identification chemistry. Org. Lett. **8**:1729.
6. **Boldt, G. E., J. P. Kennedy, M. S. Hixon, L. A. McAllister, J. T. Barbieri, S. Tzipori, and K. D. Janda.** 2006. Synthesis, characterization and development of a high-throughput methodology for the discovery of botulinum neurotoxin a inhibitors. J. Comb. Chem. **8**:513–521.
7. **Boldt, G. E., L. M. Eubanks, and K. D. Janda.** 2006. Identification of a botulinum neurotoxin A protease inhibitor displaying efficacy in a cellular model. Chem. Commun. (Cambridge) **29**:3063–3065.
8. **Breidenbach, M. A., and A. T. Brunger.** 2004. Substrate recognition strategy for botulinum neurotoxin serotype A. Nature **432**:925–929.
9. **Burnett, J. C., G. Ruthel, C. M. Stegmann, R. G. Panchal, T. L. Nguyen, A. R. Hermone, R. G. Stafford, D. G. Lane, T. A. Kenny, C. F. McGrath, P. Wipf, A. M. Stahl, J. J. Schmidt, R. Gussio, A. T. Brunger, and S. Bavari.** 2007. Inhibition of metalloprotease botulinum serotype A from a pseudo-peptide binding mode to a small-molecule that is active in primary neurons. J. Biol. Chem. **282**:5004–5014.
10. **Burnett, J. C., J. J. Schmidt, C. F. McGrath, T. L. Nguyen, A. R. Hermone, J. L. Vennerstrom, K. Kodukula, D. W. Zaharevitz, R. Gussio, and S. Bavari.** 2005. Conformational sampling of the botulinum neurotoxin serotype A light chain: implications for inhibitor binding. Bioorg. Med. Chem. **13**:333–341.
11. **Burnett, J. C., J. J. Schmidt, R. G. Stafford, R. G. Panchal, T. L. Nguyen, J. L. Vennerstrom, C. F. McGrath, D. J. Lane, E. A. Sausville, D. W. Zaharevitz, R. Gussio, and S. Bavari.** 2003. Novel small-molecule inhibitors of botulinum neurotoxin A metalloprotease activity. Biochem. Biophys. Res. Commun. **310**:84–93.
12. **Capková, K., Y. Yoneda, T. J. Dickerson, and K. D. Janda.** 2007. Synthesis and structure-activity relationships of second-generation hydroxamate botulinum neurotoxin A protease inhibitors. Bioorg. Med. Chem. Lett. **17**:6463–6466.
13. **Deshpande, S. S., R. E. Sheridan, and M. Adler.** 1995. A study of Zn-dependent metalloendopeptidase inhibitors as pharmacological antagonists in botulinum neurotoxin poisoning. Toxicon **33**:551–557.
14. **Deshpande, S. S., and R. E. Sheridan, and M. Adler.** 1997. Efficacy of certain quinolines as pharmacological antagonists in botulinum neurotoxin poisoning. Toxicon **35**:433–445.
15. **Eubanks, L. M., M. S. Hickson, W. Jin, S. Hong, C. M. Clancy, W. H. Tepp, M. R. Baldwin, C. J. Malizio, M. C. Goodnough, C. J. Dickerson, E. A. Johnson, D. L. Boger, T. J. Dickerson, and K. D. Janda.** 2007. An *in vitro* and *in vivo* disconnect uncovered through high-throughput identification of botulinum neurotoxin A antagonists. Proc. Natl. Acad. Sci. USA **104**:2602–2607.
16. **Ewing, T. J., S. Makino, A. G. Skillman, and I. D. Kuntz.** 2001. DOCK 4.0: search strategies for automated molecular docking of flexible molecule databases. J. Comput. Aided Mol. Des. **15**:411–428.
17. **Foran, P. G., B. Davletov, and F. A. Meunier.** 2003. Getting muscles moving again after botulinum toxin: novel therapeutic challenges. Trends Mol. Med. **9**:291–299.
18. **Gershon, H., and R. Parmegiani.** 1963. Antimicrobial activity of 8-quinolinol, its salts with salicylic acid and 3-hydroxy-2-naphthoic acid, and the respective copper (II) chelates in liquid culture. Appl. Microbiol. **11**:62–65.
19. **Gershon, H., and R. Parmegiani.** 1962. Antimicrobial activity of 8-quinolinols, salicylic acids, hydroxynaphthoic acids, and salts of selected quinolinols with selected hydroxy-acids. Appl. Microbiol. **10**:348–353.
20. **Gilsdorf, J., N. Gul, and L. A. Smith.** 2006. Expression, purification, and characterization of *Clostridium botulinum* type B light chain. Protein Expr. Purif. **46**:256–267.
21. **Gul, N., S. A. Ahmed, and L. A. Smith.** 2004. Inhibition of the protease activity of the LC of type A botulinum neurotoxin by aqueous extract from stinging nettle (*Urtica dioica*) leaf. Basic Clin. Pharmacol. Toxicol. **95**:215–219.
22. **Helmuth, L.** 2000. Neuroscience: an antibiotic to treat Alzheimer's? Science **290**:1273–1274.
23. **Hu, X., S. Balaz, and W. H. Shelver.** 2004. A practical approach to docking of zinc metalloproteinase inhibitors. J. Mol. Graph. Model. **22**:293–307.
24. **Jensen, M. J., T. J. Smith, S. A. Ahmed, and L. A. Smith.** 2003. Expression, purification, and efficacy of the type A botulinum neurotoxin catalytic domain fused to two translocation domain variants. Toxicon **41**:691–701.
25. **Kalb, S. R., T. J. Smith, H. Moura, K. Hill, J. Lou, I. N. Geren, C. Garcia-Rodriguez, J. D. Marks, L. A. Smith, J. L Pirkle, and J. R. Barr.** 2008. The use of Endopep-MS to detect multiple subtypes of botulinum neurotoxins A, B, E, and F. Int. J. Mass Spectrom. **278**:101–108.
26. **Kongsaengdao, S., K. Samintarapanya, S. Rusmeechan, A. Wongsa, C. Pothirat, C. Permpikul, S. Pongpakdee, W. Puavilai, P. Kateruttanakul, U. Phengtham, K. Panjapornpon, J. Janma, K. Piyavechviratana, P. Sithinamsuwan, A. Deesomchok, S. Tongyoo, W. Vilaichone, K. Boonyapisit, S. Mayotarn, B. Piya-Isragul, A. Rattanaphon, P. Intalapaporn, P. Dusitanond, P. Harnsomburana, W. Laowittawas, P. Chairangsaris, J. Suwantamee, W. Wongmek, R. Ratanarat, A. Poompichate, H. Panyadilok, N. Sutcharitchan, A. Chuesuwan, P. Oranrigsupau, C. Sutthapas, S. Tanprawate, J. Orsuwansiri, N. Phattana, et al.** 2006. An outbreak of botulism in Thailand: clinical manifestations and management of severe respiratory failure. Clin. Infect. Dis. **43**:1247–1256.
27. **Lacy, D. B., W. Tepp, A. C. Cohen, B. R. DasGupta, and R. C. Stevens.** 1998. Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. **5**:898–902.
28. **McClatchy Newspapers.** 23 July 2007, posting date. Botulism case is biggest in 30 years. Omaha World-Herald, McClatchy Newspapers, Sacramento, CA. http://www.mcclatchydc.com/homepage/story/18295.html.
29. **Moe, S. T., A. B. Thompson, G. M. Smith, R. A. Fredenburg, R. L. Stein, and A. R. Jacobson.** 2009. Botulinum neurotoxin serotype A inhibitors: small-molecule mercaptoacetamide analogs. Bioorg. Med. Chem. **17**:3072–3079.

30. **Montal, M. S., R. Blewitt, J. M. Tomich, and M. Montal.** 1992. Identification of an ion channel-forming motif in the primary structure of tetanus and botulinum neurotoxins. FEBS Lett. **313:**12–18.

31. **Montecucco, C., and G. Schiavo.** 1995. Structure and function of tetanus and botulinum neurotoxins. Q. Rev. Biophys. **28:**423–472.

32. **Park, J. G., P. C. Sill, E. F. Makiyi, A. T. Garcia-Sosa, C. B. Millard, J. J. Schmidt, and Y. P. Pang.** 2006. Serotype-selective, small-molecule inhibitors of the zinc endopeptidase of botulinum neurotoxin serotype A. Bioorg. Med. Chem. **14:**395–408.

33. **PRNewswire.** 17 April 2007, posting date. Alzheimer's drug shows promise in fighting cancer. Drugsite Trust, Auckland, New Zealand. http://www .drugs.com/clinical_trials/alzheimer-s-shows-promise-fighting-cancer-628 .html?printable=1.

34. **Ritchie, C. W., A. I. Bush, A. Mackinnon, S. Macfarlane, M. Mastwyk, L. MacGregor, L. Kiers, R. Cherny, Q. Li, A. Tammer, D. Carrington, C. Mavros, I. Volitakis, M. Xilinas, D. Ames, S. Davis, K. Beyreuther, R. E. Tanzi, and C. L. Masters.** 2003. Metal-protein attenuation with iodochlo-rhydroxyquin (Clioquinol) targeting Aβ amyloid deposition and toxicity in Alzheimer Disease, a pilot phase 2 clinical trial. Arch. Neurol. **60:**1685–1691.

35. **Schiavo, G., and M. Montecucco.** 1997. Clostridial neurotoxins, p. 169–186. In K. Aktories (ed.), Bacterial toxins: tools in cell biology and pharmacology. Chapman and Hall, Weinheim, Germany.

36. **Schiavo, G., O. Rossetto, and C. Montecucco.** 1994. Clostridial neurotoxins as tools to investigate the molecular events of neurotransmitter release. Semin. Cell Biol. **5:**221–229.

37. **Schmidt, J. J., and K. A. Bostian.** 1997. Endoproteinase activity of type A botulinum neurotoxin: substrate requirements and activation by serum al-bumin. J. Protein Chem. **16:**19–26.

38. **Schmidt, J. J., R. G. Stafford, and K. A. Bostian.** 1998. Type A botulinum neurotoxin proteolytic activity: development of competitive inhibitors and implications for substrate specificity at the S1′ binding subsite. FEBS Lett. **435:**61–64.

39. **Segelke, B., M. Knapp, S. Kadkhodayan, R. Balhorn, and B. Rupp.** 2004. Crystal structure of Clostridium botulinum neurotoxin protease in a product-bound state: evidence for noncanonical zinc protease activity. Proc. Natl. Acad. Sci. USA **101:**6888–6893.

40. **Sheridan, R. E., S. S. Deshpande, J. D. Nicholson, and M. Adler.** 1997. Structural features of aminoquinolines necessary for antagonist activity against botulinum neurotoxin. Toxicon **35:**1439–1451.

41. **Sheridan, R. E., S. S. Deshpande, and T. Smith.** 1999. Comparison of in vivo and in vitro mouse bioassays for botulinum toxin antagonists. J. Appl. Toxi-col. **19:**S29–S33.

42. **Shone, C. C., and A. K. Roberts.** 1994. Peptide substrate specificity and properties of the zinc-endopeptidase activity of botulinum type B neuro-toxin. Eur. J. Biochem. **225:**263–270.

43. **Silvaggi, N. R., D. Wilson, S. Tzipori, and K. N. Allen.** 2008. Catalytic features of the botulinum neurotoxin A light chain revealed by high resolu-tion structure of an inhibitory peptide complex. Biochemistry **47:**5736–5745.

44. **Silvaggi, N. R., G. E. Boldt, M. S. Hixon, J. P Kennedy, S. Tzipori, K. D. Janda, and K. N. Allen.** 2007. Structures of Clostridium botulinum neurotoxin serotype A light chain complexed with small-molecule inhibitors highlight active-site flexibility. Chem. Biol. **14:**533–542.

45. **Simpson, L. L., and B. R. Dasgupta.** 1983. Botulinum neurotoxin type E: studies on mechanism of action and on structure-activity relationships. J. Pharmacol. Exp. Ther. **224:**135–140.

46. **Simpson, L. L., J. A. Coffield, and N. Bakry.** 1993. Chelation of zinc antag-onizes the neuromuscular blocking properties of the seven serotypes of botulinum neurotoxin as well as tetanus toxin. J. Pharmacol. Exp. Ther. **267:**720–727.

47. **Tang, J., J. G. Park, C. B. Millard, J. J. Schmidt, and Y. P. Pang.** 2007. Computer-aided lead optimization: improved small-molecule inhibitor of the zinc endopeptidase of botulinum neurotoxin serotype A. PLoS ONE **2:**e761. doi:10.1371/journal.pone.0000761.

48. **Voigt, J. H., B. Bienfait, S. Wang, and M. C. Nicklaus.** 2001. Comparison of the NCI open database with seven large chemical structural databases. J. Chem. Inf. Comput. Sci. **41:**702–712.

49. **Yowler, B. C., R. D. Kensinger, and C. L. Schengrund.** 2002. Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I. J. Biol. Chem. **277:**32815–32819.